

## NOVEL SENSOR CONFIGURATION

### FIELD OF THE INVENTION

The present invention relates to a novel device for use in a lipid membrane biosensor,  
5 methods of preparing the device and applications thereof.

### BACKGROUND OF THE INVENTION

Biosensors based on ion channels or ionophores contained within lipid membranes that are deposited onto metal electrodes and where the ion channels are switched in the presence of analyte molecules have been described in International Patent Application Numbers  
10 WO92/17788, WO 93/21528, WO 94/07593 and U.S. Patent Numbers 5,204,239 and 6,432,629 and Australian Patent Application Numbers. 56188/94, 66063/96, 38643/95, 59925/96, 15851/97, 77509/98, 77510/98, 26283/97, 51444/93, 56403/96, 40787/89, 14657/92, 21279/88, 50334/90, 59926/96 and 65327/94 (the disclosures of which are incorporated herein by reference). As is disclosed in these applications, ionophores such as  
15 gramicidin ion channels may be co-dispersed with amphiphilic molecules, thereby forming lipid membranes with altered properties in relation to the permeability of ions. There is also disclosure of various methods of gating these ion channels (for example, the lateral segregation mechanism disclosed in International Patent Application WO90/08783) such that in response to the binding of an analyte to a binding partner attached to the membrane,  
20 the conductivity of the membrane is altered. The applications also disclose methods of producing membranes with improved sensitivity using a surface amplifier effect, and improved stability and ion flux using chemisorbed arrays of amphiphilic molecules attached to an electrode surface. The applications further disclose means of producing lipid membranes incorporating ionophores on said chemisorbed amphiphilic molecules. There is  
25 also disclosure of means of improving reproducibility, gating response towards an analyte, lateral segregation response, surface amplifier effect, and stability in serum, plasma and blood.

One difficulty observed with membrane-based biosensors is that the introduction of air to the lipid membrane surface in an uncontrolled fashion results in permanent disruption of

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membrane function due to the high interfacial energy involved. This imposes restrictions on the manufacturing process and use and application of the sensor. There is a need to provide formats for a membrane-based biosensor that stabilise the membrane to the introduction of air in an uncontrolled fashion.

## 5 SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a device comprising a well defined within a substrate, said substrate comprising, in sequence, a first base layer, a second hydrophobic layer, and a third hydrophilic layer; said well extending from the upper surface of the base layer through the second and third layers to provide an opening in the upper surface of the  
10 third layer wherein a lipid membrane comprising a closely packed array of self-assembling amphiphilic molecules extends across the well within the region defined between the first base layer and the third hydrophilic layer.

Optionally, the device further comprises a fourth hydrophobic layer wherein said well further extends to the upper surface of the fourth layer.

15 In a second aspect there is provided a method of forming a device comprising the steps of:

- (i) depositing a first base layer;
- (ii) depositing a second hydrophobic layer on the first base layer;
- (iii) depositing a third hydrophilic layer on the second hydrophobic layer to form a substrate;
- 20 (iv) forming a well in the substrate extending from the upper surface of the base layer through the second and third layers to provide an opening in the upper surface of the third layer; and
- (v) forming a lipid membrane comprising a closely packed array of self-assembled amphiphilic molecules within the well such that it extends across  
25 the well within the region defined between the first base layer and the third hydrophilic layer.

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Optionally, the method further comprises the step of depositing a fourth hydrophobic layer on the third hydrophilic layer wherein said well is formed so as to extend to the upper surface of the fourth layer.

In a third aspect there is provided a method of preparing a membrane-based biosensor comprising the steps of:

- 5 (a) adding a solution of streptavidin, avidin, neutravidin, avidin or streptavidin derivative onto the surface of a device according to the first aspect wherein the lipid membrane of said device comprises one or more biotinylated gramicidin ion channels and/or one or more biotinylated membrane spanning lipids;
- 10 (b) rinsing the device with an aqueous solution in order to remove excess streptavidin, avidin, neutravidin or other avidin or streptavidin derivative;
- (c) adding a solution of a biotinylated receptor molecule so that the receptor molecules attach to the membrane via the biotin-streptavidin-biotin link;
- 15 (d) rinsing the membrane with an aqueous solution;
- (e) removing the device from the aqueous solution and allowing to drain, such that a bead of aqueous solution is retained within the well of the device.

#### BRIEF DESCRIPTION OF THE FIGURES

20 Figure 1 shows in schematic form a well of a device according to one embodiment of the present invention.

Figure 2 shows in schematic form a well of a device according to another embodiment of the present invention in which the opening of the well is overlaid with a hydrophilic mesh.

Figure 3 shows in schematic form a device according to an embodiment of the present invention.

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**DETAILED DESCRIPTION OF THE INVENTION**

In a first aspect, the present invention provides a device comprising a well defined within a substrate, said substrate comprising, in sequence, a first base layer, a second hydrophobic layer, and a third hydrophilic layer; said well extending from the upper surface of the base layer through the second and third layers to provide an opening in the upper surface of the third layer wherein a lipid membrane comprising a closely packed array of self-assembling amphiphilic molecules extends across the well within the region defined between the first base layer and the third hydrophilic layer.

Optionally, the device further comprises a fourth hydrophobic layer wherein said well further extends to the upper surface of the fourth layer.

Preferably, the lipid membrane is composed such that the impedance of the membrane is dependent on the presence or absence of an analyte to be detected. The composition of such membranes are described in detail in the publications referred to herein.

The present inventors have found that the device including the features of the first aspect can retain a protective bead of a polar liquid, such as water or an aqueous solution, on the membrane surface thereby stabilising the membrane to the introduction of air.

The dimensions of the well are preferably selected such that the bead of retained polar liquid is of sufficient size to prevent contact of the membrane with air, but still be capable of rapid exchange with analyte solutions or other solutions. Examples of suitable dimensions are: for the first layer, 50 to 150 nm, preferably 100 nm; for the second layer, 100 to 300 nm, preferably 250 nm; for the third layer, 400 to 600 nm, preferably 500 nm; and for the optional fourth layer, 100 to 300 nm, preferably 200 nm. Preferably, the opening of the well is substantially circular with a diameter of from about 10 to 200 microns, more preferably 20 to 150 microns and most preferably 100 microns.

Preferably, the first base layer is a conductive layer. This enables the device to act as a component in an electrode sensor using, eg, the ion channel switches known in the art. The conductive layer may be formed from any conductive material capable of acting as electrode including gold, silver, copper, conducting polymers and the like. Gold is particularly preferred.

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The third hydrophilic layer comprises a hydrophilic material. The hydrophilic material may be of any suitable type which has some affinity for the polar liquid and is preferably metallic, ceramic or polymeric.

5 It is further preferred that the hydrophilic material is selected from the group consisting of silicon carbide, silicon oxide, silicon dioxide, titanium dibromide, titanium oxide, titanium nitride, zinc oxide, zirconium dioxide, magnesium oxide, iron oxides, graphite, boron nitride, chromium nitride, and poly vinylidene fluoride. Titanium oxide is particularly preferred.

10 The second and optional fourth hydrophobic layers may be formed from any suitable hydrophobic material that resist wetting by the polar liquid. The materials to form the second and optional fourth layers may be the same or different.

15 It is preferred that the hydrophobic material is an organic polymer and/or is selected from the group consisting of polyamides, PVC, polystyrenes, polyesters, polycarbonates, polyurethanes, nylons Glass fibre, Plastics, Silicon rubbers, Latex, glass, vinyl, phenolic, resins, brass, Tetrafluoroethylene Octadecyltrichlorosilane, Teflon, Silicon nitride, Silicon carbide, aluminium nitride, oxidised silicon carbide, Butadiene Styrene, Ethylene vinyl acetate, and PTFE (polytetrafluoroethylene) polymer. It is preferred that the hydrophobic material is oxidised silicon carbide or aluminium nitride.

20 In a further preferred embodiment the third hydrophilic layer comprises titanium oxide and the second hydrophobic layer comprises oxidised silicon carbide.

In a preferred form, the internal circumference of the well is varied such that it is greater in the region defined by the fourth hydrophobic layer than in the region defined by the second hydrophilic layer.

25 In a further preferred form, a mesh covers at least a portion of the well opening. This allows the well to retain the polar liquid more tenaciously by increasing the capillary force and therefore allows for the size of the well and hence the size of the electrode to be maintained. In a more preferred embodiment, the mesh is formed as an extension of the optional fourth hydrophobic layer.

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The mesh may form any tessellating pattern, such as rectangular, square, triangular, hexagonal and the like. A hexagonal pattern is particularly preferred. The wall partitions of the mesh are preferably from 1 to 10 nm in thickness and the individual cells are preferably from 20 to 100 nm wide at their widest point.

- 5 It would be understood by those skilled in the art that sensor according to the present invention may include a plurality of wells in a single substrate thus allowing for an array of the wells to be formed.

Turning to Figure 1, a portion of a device including the features of the present invention is depicted. The sensor comprises a laminar substrate comprising a silicon support 1, a  
10 titanium layer 2 of about 5 nm thickness, a first conductive base layer 3 of gold of about 100 nm in thickness, a second hydrophobic layer 4 of silicon nitride of about 200 nm in thickness; a third hydrophilic layer 5 of silicon oxide of about 500 nm in thickness, and a fourth hydrophobic layer 6 of about 200 nm thickness. A well 9 is defined within the  
15 substrate such that it extends from the upper surface of the base layer through the second, third and fourth layers to provide a well opening 10 in the upper surface of the fourth layer. A membrane is located within the region of the well defined by the second hydrophobic layer, the membrane comprising a lower first layer 7 and an upper second layer 8 of closely packed amphiphilic molecules and a plurality of ionophores with at least a proportion of the molecules and ionophores of the lower first layer 7 being connected to the upper surface of  
20 the first conductive layer 3 by means of linker groups.

As can be seen, the internal circumference of the well is varied such that it is greater in the region defined by the fourth hydrophobic layer 6 than in the region defined by the second hydrophobic layer 4.

Turning to Figure 2, a top view of four wells 11 in a substrate is displayed in which the  
25 openings of the wells are covered with a hexagonal mesh 12. The mesh in this case is silicon dioxide. A further layer of silicon nitride 13 coats the remainder of the substrate surface.

Figure 3 displays the membrane-based biosensor of Figure 1 in which a bead of liquid 14 is trapped in the well above the surface of the membrane by the arrangement of hydrophilic and hydrophobic layers.

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In a second aspect there is provided a method of forming a device comprising the steps of:

- (i) depositing a first base layer;
- (ii) depositing a second hydrophobic layer on the first base layer;
- 5 (iii) depositing a third hydrophilic layer on the second hydrophobic layer to form a substrate;
- (iv) forming a well in the substrate extending from the upper surface of the base layer through the second and third layers to provide an opening in the upper surface of the third layer; and
- 10 (v) forming a lipid membrane comprising a closely packed array of self-assembled amphiphilic molecules within the well such that it extends across the well within the region defined between the first base layer and the third hydrophilic layer.

Optionally, the method further comprises the step of depositing a fourth hydrophobic layer on the third hydrophilic layer wherein said well is formed so as to extend to the upper  
15 surface of the fourth layer.

Preferably, the first base layer is deposited on a layer of titanium of a support material comprising a silicon support and the layer of titanium. More preferably, the support material is formed by depositing a layer of titanium on the silicon support. Even more preferably, the silicon support is a single crystal silicon wafer.

20 Preferably, the first base layer is from 50 nm to 150 nm thick, more preferably 100 nm thick. Preferably, the second hydrophobic layer is from 100 nm to 300 nm thick, more preferably 200 nm thick. Preferably, the third hydrophilic layer is from 400 nm to 600 nm thick, more preferably 500 nm thick. Preferably, the optional fourth hydrophobic layer is from 100 nm to 300 nm thick, more preferably about 200 nm thick. Preferably, the opening of the well is  
25 substantially circular with a diameter of from about 10 to 200 microns, more preferably 20 to 150 microns and most preferably 100 microns.

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Preferably, the first base layer is a conductive layer, more preferably the conductive layer is formed from gold.

The third hydrophilic layer comprises a hydrophilic material. The hydrophilic material may be of any suitable type which has some affinity for the polar liquid and is preferably  
5 metallic, ceramic or polymeric.

It is further preferred that the hydrophilic material is selected from the group consisting of silicon carbide, silicon oxide, silicon dioxide, titanium dibromide, titanium oxide, titanium nitride, zinc oxide, zirconium dioxide, magnesium oxide, iron oxides, graphite, boron nitride, chromium nitride, and poly vinylidene fluoride. Titanium oxide is particularly  
10 preferred.

The second and optional fourth hydrophobic layers may be formed from any suitable hydrophobic material that resist wetting by the polar liquid. The materials to form the second and optional fourth layers may be the same or different.

It is preferred that the hydrophobic material is an organic polymer and/or is selected from  
15 the group consisting of polyamides, PVC, polystyrenes, polyesters, polycarbonates, polyurethanes, nylons Glass fibre, Plastics, Silicon rubbers, Latex, glass, vinyl, phenolic, resins, brass, Tetrafluoroethylene Octadecyltrichlorosilane, Teflon, Silicon nitride, Silicon carbide, aluminium nitride, oxidised silicon carbide, Butadiene Styrene, Ethylene vinyl acetate, and PTFE (polytetrafluoroethylene) polymer. It is preferred that the hydrophobic  
20 material is oxidised silicon carbide or aluminium nitride.

Preferably, the well is formed by etching.

Preferably, the lipid membrane comprises a lower first membrane layer and an upper second membrane layer and wherein the lipid membrane further comprises a plurality of ionophores with at least a proportion of the molecules and ionophores of the lower first  
25 layer being connected to the upper surface of the first base layer by means of linker groups.

More preferably, the step of forming the lipid membrane comprises: forming a first solution containing one or more amphiphilic molecules, one or more linker groups and one or more ionophores in a first organic solvent (preferably ethanol); contacting the first base layer of the well with the first solution to form the lower first membrane layer comprising a closely



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packed array of said amphiphilic molecules and said ionophores wherein said lower first membrane layer is connected to the first base layer by means of said linker groups; rinsing the device with a suitable second organic solvent (preferably ethanol); removing the excess second organic solvent; forming a second solution of one or more amphiphilic molecules and one or more ionophores in a suitable third organic solvent (preferably ethanol);  
5 contacting the second solution with the device comprising said first lower membrane layer to form the second layer membrane layer; rinsing the device with an aqueous solution; and removing the device from the aqueous solution and allowing to drain.

Preferably, said second organic solvent is removed by rapid air drying.

10 Preferably, immediately upon removal of the excess second organic solvent, the device is immersed in the third solution.

Preferably, the one or more ionophores comprise gramicidin A or an analogue thereof. More preferably, the one or more ionophores are biotinylated.

15 Preferably, one or more receptors are attached to the surface of the membrane. More preferably, the one or more receptors are attached to the membrane by using streptavidin, avidin or one of the related biotin binding-proteins. Even more preferably, one or more receptors are coupled to one or more biotinylated gramicidin ion channels and/or to one or more biotinylated membrane-spanning lipid.

20 In a third aspect there is provided a method of preparing a membrane-based biosensor comprising the steps of:

(a) adding a solution of streptavidin, avidin, neutravidin, avidin or streptavidin derivative onto the surface of a device according to the first aspect wherein the lipid membrane of said device comprises one or more biotinylated gramicidin ion channels and/or one or more biotinylated membrane spanning lipids;

25 (b) rinsing the device with an aqueous solution in order to remove excess streptavidin, avidin, neutravidin or other avidin or streptavidin derivative;

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- (c) adding a solution of a biotinylated receptor molecule so that the receptor molecules attach to the membrane via the biotin-streptavidin-biotin link;
  - (d) rinsing the membrane with an aqueous solution;
  - (e) removing the device from the aqueous solution and allowing to drain, such that a bead of aqueous solution is retained within the well of the device.
- 5                    Preferably, the electrode is stored at between minus 20°C and plus 5°C.

The preparation of a device in accordance with an embodiment of the present invention will now be described. The embodiment is concerned with device that can be used as a component in an electrode sensor that detects the presence of an analyte by an adjustment in the conductivity of the membrane. It would be clear to a person skilled in the art, however, that devices and methods of the present invention also extends to other techniques for detecting the presence of an analyte using a device such as by using fluorescence techniques and the like.

A device according one embodiment to the present invention comprising a substrate having a well defined therein can be prepared by:

- (1) Taking a support material and depositing on it in sequence a layer of titanium typically 5nm in thickness, a layer of gold typically 100 nm in thickness which forms an electrode of the membrane-based biosensor, a hydrophobic layer typically 200 nm in thickness, a hydrophilic layer typically 500 nm in thickness, and optionally a hydrophobic layer typically 200 nm in thickness to form a laminar substrate; and
- (2) Etching the substrate to form a well or wells with the required geometry; and
- (3) Cleaning the etched substrate and reducing gold oxides on the surface.

It is preferred that the support material is a single crystal silicon wafer.

It is preferred that the electrode area is wet etched using a photolithographic patterning approach.

It is preferred that the gold electrode consists of a freshly evaporated or sputtered gold electrode.

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A membrane can then be formed in the well of the device by:

- (4) Forming a solution containing amphiphilic molecules, linkers and ionophores; and
  - (5) Contacting the cleaned gold base of the well with the solution, to form a first layer membrane comprising a closely packed array of amphiphilic molecules and a plurality of ionophores, the first layer membrane being connected to the electrode by means of a linker group;
  - (6) Rinsing the wafer with a suitable organic solvent; and
  - (7) Removing the excess organic solvent used for rinsing;
  - (8) Forming a solution of lipid and a plurality of ionophores, dispersed in a suitable solvent; and
  - (9) Contacting the solution with the electrode containing a first layer membrane to form a second layer membrane as described in the prior art; and
  - (10) Rinsing the electrode surface with an aqueous solution; and
  - (11) Removing the electrode from the aqueous solution and allowing to drain.
- 15 The membrane so formed extends across the well within the region defined between the first base layer and the third hydrophilic layer.
- It is preferred that the solvent for the adsorbing solutions in steps (4) and (8) and for the rinsing step (6) is ethanol. It is further preferred that in step (6) the solvent is removed by rapid air drying.
- 20 It is preferred that immediately upon removal of excess solvent in step (7) the electrode is immersed in the solution described in step (8).
- It is preferred that the ionophore in step (8) is gramicidin A or an analogue thereof. It is further preferred that this molecule is biotinylated to enable subsequent binding of streptavidin or analogues thereof.

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It is preferred that rinsing step (10) is carried out before the solvent drains from the bilayer membrane formed in step (9).

Once the membrane has been formed, the membrane can be further functionalised in order to provide for the detection of the presence of analyte by the membrane-based biosensor.

5 One convenient method to attach appropriate receptors to the surface of a membrane is by using streptavidin, avidin or one of the related biotin binding-proteins as a means of coupling a wide range of receptors onto a biotinylated gramicidin ion channel or membrane-spanning lipid. An example of such a process comprises the steps of:

- 10 (a) Adding a solution of streptavidin, avidin, neutravidin, avidin or streptavidin derivative onto the surface of the membrane of a membrane-based biosensor according to the present invention in which at least a portion of the components are biotinylated;
- (b) Rinsing the electrode with an aqueous solution in order to remove excess streptavidin, avidin, neutravidin or other avidin or streptavidin derivative;
- 15 (c) Adding a solution of a biotinylated receptor molecule so that the receptor molecule is attached to the membrane via the biotin-streptavidin-biotin link; and
- (d) Rinsing the coated electrode with an aqueous solution; and
- (e) Removing the electrode from the aqueous solution and allowing to drain, such that a bead of water is retained within the well of the device; and
- 20 (f) Storing the electrode at reduced temperature. It is preferred that the electrode is stored at between minus 20°C and plus 5°C.

It is preferred that the biotinylated receptor molecules are introduced into the well of the device using an ink jet robot.

25 It can be seen from the example above of attaching biotinylated receptor molecules to the surface of the membrane that the ability to retain liquid on the surface of the membrane in a controlled fashion enables fabrication using a simple sequential dipping technology. This is highly advantageous in terms of the reduction of manufacturing costs by removing the

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requirement for the complex liquid handling systems which are otherwise necessary to avoid the formation of a membrane-disrupting air-liquid interface during the manufacturing process.

5 This simple sequential dipping technology allows for a desired sensor configuration to be rapidly assembled by combining a stored membrane with a an appropriate receptor solution. This process, combining previously prepared and stored components, allows for simplified fabrication which can be carried out remotely from the point of manufacture.

10 Further, as a consequence of the increased retention of the bead of liquid provided by the present invention, it is envisaged that the membrane of a device of the present invention may be dried in a relatively controlled fashion such that the lipid membrane retains its function, structure and activity when resolvated. This will assist in the storage and handling of devices according to the present invention.

15 It is preferred that in the drying process the amount of liquid retained above the membrane be reproducibly and precisely controlled, hence methods of drying such as lyophilisation, evaporation, or evaporation over controlled humidity, are preferred.

20 The devices of the present invention provide further advantages in simplifying the analyte detection process in that it allows for the use of air bubbles to separate different components of a liquid flow stream. The use of air to separate different components of a liquid flow stream and prevent their mixing is well known in the art, but has not previously been applicable to lipid membrane sensors due to the possibility of permanent disruption of the membrane. The devices of the invention provide a membrane which is protected from the effects of the uncontrolled introduction of air or gas by the presence of a trapped bead of polar liquid or solvent. By retaining liquid on the lipid membrane surface even in the presence of air, the present invention allows, for example, the sequential passage of rinse, calibration and analyte solutions over the membrane with each solution being separated from the next by interposition of an air bubble. This avoids the requirement for complex liquid handling systems or procedures which are otherwise necessary to avoid mixing and hence cross-contamination of the different components during the analytical procedure. As will also be readily apparent to those skilled in the art, this decreased susceptibility to the presence of air allows for a more rugged assay procedure. It is expected that the present

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invention will be more tolerant to the presence of transient air bubbles in the test solutions, eliminating the need to treat samples or introduce procedures to remove air from the system.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

All publications mentioned in this specification are herein incorporated by reference. Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed anywhere before the priority date of each claim of this application.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.